Molecular Characterization of Nonpolypoid Colorectal Adenomas

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Chapter 1

General Introduction

CRC GENERAL

Colorectal cancer (CRC) is a major healthcare problem in the Western world. It ranks as the second most common cause of cancer-related death in the Western world and in the Netherlands each year 13000 new patients are diagnosed and 5000 die from the disease[1] (www.cijfersoverkanker.nl). CRC can be subdivided into sporadic and familial cases, the majority of CRCs (~80%) are sporadic cases and approximately 20% of all CRCs are attributable to a familial (hereditary) disorder. However, in only about 5% of all familial CRC cases the causative genetic defect is identified, including Familial Adenomatous Polyposis (FAP),[2] Lynch syndrome (also known as hereditary non-polyposis colon cancer (HNPCC)[3] and MUTYH-associated polyposis (MAP)).[4] Other even more rare hereditary disorders are juvenile polyposis, Peutz-Jeghers syndrome and hyperplastic polyposis syndrome (HPS).

Although rare, the above mentioned hereditary disorders are used as models to study CRC carcinogenesis. Based on these models major advances have been made regarding our understanding of the molecular events leading to CRC and which potential pathways involving different lesion types are implicated. The causes of sporadic colorectal cancer are still not completely understood, but inflammatory bowel disease,[5,6] environmental and lifestyle factors are believed to increase the risk of developing the disease.[7-9]

EVOLUTION OF THE COLORECTAL CARCINOGENESIS MODEL

Polyp-cancer sequence (model)

All carcinomas (i.e. malignant tumors of epithelial origin) are thought to pass through an intermediate stage which lies between normal epithelium and invasive carcinoma. In the large intestine this premalignant stage is called dysplasia and the premalignant lesions are called adenomas. Adenomas arise from mucosal epithelium as a failure in the regulation of normal processes like proliferation, differentiation and apoptosis (programmed cell death). This failure leads to an expansion of the proliferative compartments from the lower part to the upper part of the crypt, where undifferentiated cells accumulate at the luminal surface. In the large intestine the concept of precursor lesions has long been dominated by the “polyp-cancer” or adenoma-carcinoma sequence which was coined by Morson in 1974.[10] In his concept Morson described that most (if not all) colorectal carcinomas develop from polyps and moreover that these polyps have a malignant potential. However, not all polyps evolve into a malignant lesion. Morson used the term polyp as synonym for adenoma and even today these terms are used interchangeable, ignoring the existence of nonpolypoid adenomas.

The concept that not all adenomas become malignant is still contemporary. Today, based on the incidence of finding a focus of cancer in adenomas, it is estimated that only 5% of all adenomas will progress into a carcinoma.[11] Size, villous architecture and grade of dysplasia[12] were

found by Morson and colleagues to be associated with the chance of finding a focus of carcinoma in a (polypoid) adenoma. Nonetheless these variables are not 100% predictive for malignant progression. The term “advanced adenoma” (i.e. an adenoma larger than 1 cm and/or with a villous component and/or with high-grade dysplasia) to identify adenomas at high risk of progression to cancer, is based on over-interpretation of the Morson data.[13,14] Formal evidence for the predictive value of these parameters hardly exists, since a longitudinal study would be ethically impossible to perform. Currently, the term “advanced adenoma” is widely used in the clinic to determine the surveillance regimen after colonoscopy.

Two interesting aspects of the adenoma-carcinoma (polyp-cancer) sequence are that, first if adenomas are the precursors of carcinomas, removing them will prevent death from colorectal cancer. Second, the polyp-cancer sequence provides clear premalignant and malignant phenotypes that can be studied and that can be correlated to genetic alterations in order to get more insights in the carcinogenesis of colorectal cancer.

Adenoma-carcinoma sequence (molecular)

Around 1990 Vogelstein and colleagues provided a molecular basis to the adenoma-carcinoma sequence, popularly called the “Vogelgram”.[15,16] Using colorectal lesions of different stages Vogelstein and colleagues created a model showing that CRC is a genetic disease marked by the accumulation of genetic changes. This multistep model indicates the crucial molecular events that are taking place during the progression from normal colorectal epithelium via adenomas to carcinoma.

Three main events during this progression were described. The first is the formation of an adenoma. Around that time, mutations in adenomatous polyposis coli (*APC*) were identified as the cause of FAP as well as an early event in sporadic CRC.[17,18] Inactivation of *APC* leads to the disruption of the Wnt-pathway, which gives rise to the formation of an adenoma. Accumulation of more alterations such as *KRAS* mutations[19] and losses of chromosome 5, 17 and 18, were associated with the second event, growth of the adenoma. Inactivation of TP53, located on chromosome 17p, which was identified in 1989 as a tumour suppressor gene and shown to be mutated in a high percentage of many cancers including CRC,[20] mediated the third event in the adenoma to carcinoma progression.

The model of Vogelstein describes colorectal carcinogenesis as a sequential step wise process from normal mucosa to a malignant lesion and suggests that colorectal carcinogenesis is a single pathway. However, already at publication of the model it was stated that the accumulation of genetic changes rather than their order seems to be the major determinant factor of neoplastic changes.[16] Over the years the Vogelgram provided a framework to gain more understanding of the initiation and progression of CRC and underlined the importance of the cumulative accumulation of genetic alterations. Although the Vogelgram has been of great value, as recently confirmed by the Cancer Genome Atlas Group,[21] it is becoming more and more clear that it does not cover the

complete spectrum of colorectal carcinogenesis. In fact only a few CRCs actually evolve along this pathway.[22,23]

CARCINOGENIC ALTERATIONS

Oncogenes and tumor suppressors

Colorectal carcinogenesis is driven by aberrant functioning of genes that regulate a variety of biological processes including cell proliferation, apoptosis and angiogenesis.[24] Genes that promote tumor formation are called oncogenes, whereas genes inhibiting tumor formation are called tumor suppressor genes. Activation of oncogenes and inactivation of tumor suppressor genes during carcinogenesis can be achieved through several mechanisms.

1) Mutations, alterations in the nucleotide sequence of a cell. When these alterations occur in a coding region, a gene, this can lead to altered protein products or altered protein amounts. Mutations involved in CRC carcinogenesis include inactivation mutations in tumor suppressor genes, such as *TP53*, *APC*, as well as activating mutations in oncogenes, including *KRAS*, *BRAF* and *EGFR*.

2) Complex numerical and structural chromosomal alterations are common in solid tumors and can lead to altered expression of oncogenes and tumor suppressor genes. Numerical alterations include polyploidy and aneuploidy. Polyploidy is an exact multiplication of the normal DNA content in a cell of two copies per chromosome. Aneuploid cells contain a varying number of copies of each chromosome. Structural rearrangements can be balanced or unbalanced, the latter leading to loss or gain of parts of the genome. Gains of chromosomal regions can result in increased expression of oncogenes located at those regions. Similarly, chromosomal losses may lead to decreased expression of tumor suppressor genes. Studying these chromosomal gains and losses can lead to the identification of genes relevant to CRC carcinogenesis.[25-27]

3) Loss of heterozygosity (LOH) is the loss of one of the two alleles at one or more loci in a cell. It is a measure of genetic instability and can result in altered gene expression or altered gene products. LOH reflects allelic imbalance caused by somatic recombination. Whereas in normal cells paternal and a maternal allele of each gene is present, somatic recombination can result in loss of one of these alleles, while leaving the chromosome itself intact. Loss of heterozygosity due to loss of one parental copy in a region is also called hemizygosity. Here, the gene in question is not completely inactivated but rather a dose effect is established.

4) Epigenetic changes do not alter the DNA sequence itself, but rather the accessibility of the DNA for transcription factors, thereby influencing gene expression. This is achieved by modification of either bases in the DNA sequence or the histone proteins, around which the DNA is wrapped. These modifications include methylation, ubiquitination, phosphorylation and acetylation. Nowadays, it is clear that epigenetic deregulation of gene expression contributes to carcinogenesis

in general.[28] The most common investigated epigenetic alteration in CRC is DNA promoter hypermethylation. This occurs at CpG dinucleotide-dense regions, called CpG islands and can lead (methylation-mediated) silencing of the downstream located gene (e.g. a tumor suppressor gene).

GENOMIC INSTABILITY PATHWAYS

The Vogelgram postulated the importance of genomic instability (chromosomal instability, LOH, gene mutations etc) in colorectal carcinogenesis. Currently, it is clear that CRC develops through multiple pathways resulting in deregulation of a variety of biological processes. This requires multiple genetic events, which lead to aberrant activation or inactivation of these biological pathways.[24] A genetically unstable environment, which occurs early in tumorigenesis, is a condition for adenoma to carcinoma progression.[29,30] In CRC two forms of genetic instability are recognized; microsatellite instability (MSI)[31] and chromosomal instability (CIN).

Microsatellite instability

With the publication of the Vogelgram a search for novel tumor suppressors in CRC was initiated, which was accelerated by the invention of a new technique called polymerase chain reaction (PCR).[32,33] During this search a potentially new CRC pathway was found by Perucho *et al*.[34] CRCs evolved by this pathway contained less *KRAS* or TP53 gene mutations. Moreover, this new type of CRC was more common in the proximal colon, less likely to be invasive and suggested to be hereditary. Later on this new pathway was named microsatellite instability (MSI).[35]

MSI tumors are often diploid or near-diploid at the chromosomal level and harbor frequent alterations in short repetitive nucleotide sequences, called microsatellites. The underling cause for MSI is inactivation of the DNA mismatch repair (MMR) system, by inactivation of the MMR genes, such as MLH1, MSH2 and MSH6. By a failing MMR system errors occur during DNA synthesis, which can lead to mutations in coding genes thereby affecting the expression or function of the gene (e.g. tumor suppressor gene or oncogene).

MSI is observed in almost all carcinomas from patients with Lynch syndrome. These patients harbor a germline mutation in one of the MMR genes. In sporadic colorectal carcinomas about 15% shows MSI.[36] In these patients inactivation of the MMR system is frequently caused by promoter hypermethylation mediated silencing of *MLH1*.

Interestingly, some typical features of MSI carcinomas were already noticed by Perucho, including the frequent proximal location and a better prognosis in general. Moreover these tumors have a poor or mucinous differentiation and contain more tumour-infiltrating lymphocytes.[35,37] This infiltrate potentially results from an immune reaction against neoantigens formed as a result of frameshift mutations in protein-coding sequences.[38]

Chromosomal instability

CIN is found in approximately 85% of colorectal carcinomas and is marked by DNA copy number alterations and structural rearrangements (aneuploidy). In 1890 Von Hansemann was the first to describe this phenomenon in cancer cells by observing that these cells underwent asymmetric mitoses.[39] In 1914 Boveri hypothesized that cancer was the result of defects in the genetic material.[40] Although this phenomenon was already described over a century ago, the mechanism behind chromosomal alterations is still not clarified. It is thought that defects in DNA replication checkpoints and in processes that control chromosome segregation during mitosis, i.e. mitotic-spindle checkpoints, are accountable for chromosomal instability.[41-43]

With the development of the chromosomal banding technique,[44,45] chromosomal deletions, gains and translocations could be found and described in greater detail. The first chromosomal translocation described was the Philadelphia chromosome in chronic myeloid leukemia[46] which turned out to be a translocation between chromosomes 9 and 22.[47]

With the development of comparative genomic hybridization (CGH)[48,49] and later microarray based CGH (arrayCGH),[50,51] it became possible to visualize all chromosomal numerical alterations in the whole (cancer) genome in one experiment. A considerable amount of studies used this technique to investigate a wide range of cell lines and (solid) tumors, including CRC.

The first studies in CRC using CGH, confirmed the loss of chromosome 18q already described by the Vogelgram[15,16] but also revealed frequent gains of chromosome 20.[52,53] In the last two decades the amount of (array)CGH studies has rapidly increased and demonstrated the occurrence of many chromosomal alterations in colorectal carcinogenesis, including losses of chromosome 1p, 4q, 5q, 8p, 14q, 15q, 17p, 18pq, 21q, and 22q and gains of chromosomes 1q, 7pq, 8q, 11q, 12p, 13q, 16pq, 19q and 20pq.[54-56]

Because (array)CGH analysis is also possible on archival or formalin-fixed paraffin-embedded (FFPE) material, this provided the opportunity to study tumors with clinical follow-up data enabling the identification of genetic changes that are related to prognosis. Deletion of chromosome 4p has been associated with relapse,[57,58] whereas loss of chromosome 18 was associated with response to therapy[59] as well as the transition from adenoma to carcinoma.[60]

Interestingly, some of the DNA copy number alterations found in carcinomas were already detectable in adenomas as well.[56,61,62] Hermsen *et al* found that losses of 8p21-pter, 15q11-q21, 17p12-13, and 18q12-21, and gains in 8q23-qter, 13q14-31, and 20q13 were strongly associated with adenoma to carcinoma progression, independent of the degree of dysplasia.[61]

The chromosomal alterations that are observed in CIN tumors are often coinciding with a set of mutations in specific tumor suppressor genes and oncogenes. These mutations activate or deactivate pathways that are critical for CRC initiation and progression. It remains to be elucidated whether the chromosomal alterations create a perfect environment for these mutations to occur

or vice versa, where these mutations create a perfect environment for chromosomal instability to occur, similar to the mechanism that causes MSI.[63] Perhaps this question can be answered in the coming decade with the help of a recently developed technique called next generation sequencing (or massively parallel sequencing). This technique enables simultaneously determination of numerical and structural chromosomal changes as well as mutations of all coding (genes) and non-coding regions of the genome. The big challenge for the coming years is the analysis/interpretation of this tremendous amount of data. A first attempt has already resulted in novel mutations and recurrent chromosomal translocations.[21]

CpG ISLAND METHYLATION

In addition to genetic alterations (such as mutations in tumor suppressor genes or oncogenes, LOH and chromosomal alterations) there are also epigenetic alterations. These can be histone modification as well as DNA methylation. Together these genetic and epigenetic alterations interact in driving the development of cancer.[64] The most common investigated epigenetic alteration in CRC is CpG island promoter hypermethylation, leading (methylation-mediated) silencing of the downstream located gene (e.g. a tumor suppressor gene). This hypermethylation has been shown to be important in the initiation and progression of CRC[65-68] and almost all CRCs show to some extent CpG island promoter methylation.

A subset of adenomas and carcinomas showed significantly more promoter methylation than others, reason why some authors refer to this as a so called third colorectal pathway (next to MSI and CIN) named CpG island methylation phenotype (CIMP) pathway.[69-71] Tumors affected by this phenotype are characterized by a high degree of concordant CpG island methylation. CIMP colorectal tumors differ from non-CIMP tumors in their pathological and molecular profiles. These tumors are overrepresented in proximal tumors of the colon, occur more often in women and tend to occur in older patients.[72-75] At the molecular level these tumors are strongly associated with mutations in *BRAF* or *KRAS* and they have been suggested to be associated with a serrated phenotype (see paragraph 4).

Overall, 30% to 50% of colorectal cancers fulfill the criteria for CIMP[76-78] and consequently this group is (partly) overlapping with other genetic pathways, CIN and especially with MSI. There is a strong link between MSI and CIMP, and there are claims that the CIMP characteristics do not represent a distinct phenotype in CRC but rather reflects those of MSI tumors.[79,80] However, CIMP is also associated with distinct features in cases without MSI.[81]

Yet, it remains to be resolved wheter CIMP tumors represent a separate biological entity. It has been suggested that activating mutations in methylating enzymes (DNA methyl transferases (DNMTs)) or alterations in genes that control mechanisms normally protecting DNA from aberrant methylation may be a possible cause of CIMP[82] and that epigenetic and genetic events simultaneously

contribute to tumor progression. An alternative hypothesis for the occurrence of CIMP tumors is that CIMP reflects a chronic exposure to epimutagens chemicals that induce epigenetic abnormality that could cause or accelerate cancer development through epigenetic pathways.[69,82]

A key issue of CIMP tumors is the lack of a uniform definition of CIMP and differences in data interpretation.[83] Consequently, this has led to many discussions whether the detection of CIMP is dependent on the markers analyzed, experimental tools used and type of data analysis. In addition CIMP may represent a phenomenon associated with aging.[69,80,84-86]

SERRATED PHENOTYPE

Serrated lesions of the large intestine represent a heterogeneous group of lesions that share a phenotypic characteristic. The epithelial layer of these lesions under the microscope reveals a saw tooth or "serrated" pattern.

Ordinary and innocent hyperplastic polyps are by far the most common serrated lesions. These hyperplastic polyps, especially in the rectum, are usually small (< 0.5 cm) and show a characteristic serrated or saw-tooth appearance of the upper part of the crypts with clear mucin production. The nuclei are smallest in the superficial part of the crypt and show no stratification or hyperchromatism, like nuclei in adenomas.[87]

Less common variants include traditional serrated adenomas, mixed adenomas and so called sessile serrated lesions, also referred to as sessile serrated adenomas or sessile serrated polyps. These are particularly interesting, they also have a serrated epithelium, but do not show nuclear atypia and hence no dysplasia and they mostly are flat or slightly elevated, but not polypoid. Typically crypts with a wider basis ("boot like") can be recognized. These lesions have been associated with *BRAF* mutations and MSI colorectal cancer and are typically right sided and difficult to recognize. The frequency of these lesions in most series does not exceed 5%.[87]

Recently, serrated adenomas have been suggested to be the precursor lesions of sporadic MSI CRCs. This hypothesis is based on the observation that sporadic MSI CRCs infrequently harbor mutations in *APC*, *KRAS* or TP53, mutations which are typically found in conventional adenomas but not in serrated lesions.[88-90] Sporadic MSI CRCs, on the other hand, frequently harbor mutations in *BRAF* and show CIMP which are both infrequently found in conventional adenomas but frequent in serrated lesions.[76,78,91-93] In hereditary Lynch syndrome CRCs, which have a high frequency of MSI, the *BRAF* mutations and CIMP phenotype are infrequently present.[91,94,95] This would place the serrated adenomas as the precursor lesions of sporadic MSI CRC, which account for about 15% of all colorectal cancers.

However, this hypothesis await further evidence before a definite conclusion can be reached. This is further complicated by different interpretations of the morphological features of

serrated polyps. Even among expert GI-pathologists there is significant inter-observer variability in classification creating an extra barrier to investigate the molecular and clinical characteristics of these lesions.[96,97]

NONPOLYPOID ADENOMAS

As described before the adenoma-carcinoma sequence has provided the basis for the paradigm of multistep carcinogenesis. Particularly after Vogelstein and colleagues described the association of *APC* mutations, *KRAS* mutations, 18q loss and *TP53* loss of function with this adenoma-carcinoma sequence. Whereas the terms polyps and adenomas have long been used as synonyms, in 1985 Muto *et al* already described a type of lesion in the colorectum that was termed 'small flat adenoma'.[98] As paradigms usually leave little room for nuances, the notion that nonpolypoid precursors of sporadic colorectal cancer could exist has largely been ignored. Consequently, observations made in Japan of the undisputable existence of nonpolypoid colorectal neoplasms (NP-CRNs) were long interpreted as reflecting a non-Western entity.[99] For the GI community at large this has changed only recently with publications on large US based series of NP-CRNs[100] and it is now well accepted that phenotypically different types of colorectal lesions exist.

Currently it is unclear how the different molecular pathways, described above, are associated with the morpholocially distinct lesions that are seen in the colorectum.

Detection and classification

For obvious reasons NP-CRNs are more difficult to detect, in particular when endoscopists have a low level of awareness of these subtle lesions, or in case of suboptimal bowel preparation. In addition, NP-CRNs are more prevalent in the proximal colon[101] which can be more difficult to inspect by colonoscopy.

Whereas previously in the West the prevalence of nonpolypoid lesions has been unrightfully underestimated, in Japan nonpolypoid lesions have been reported to represent 12-40% of colorectal adenomas or early carcinomas.[102,103] With Japanese endoscopists collaborating with Western colleagues, knowledge and expertise on recognition and diagnosis of NP-CRNs have spread, and similar prevalences of nonpolypoid lesions in the West are now reported.[100,104-108] The current method to detect NP-CRNs is by selective chromoendoscopy, which means that a suspected area of mucosa is dye-sprayed (methylene blue, crystal violet or indigo carmine). This dye-spraying has been suggested to enhance the detection rate of NP-CRNs[109] and enables the endoscopist to better indicate the margins of the lesion and completely remove the lesions.

Together with increasing awareness and recognition of nonpolypoid lesions, the nomenclature around nonpolypoid lesions/NP-CRNs has evolved. Currently, it is discouraged to use the term

flat lesion, as this definition is not well described. The preferred term to use for these lesions is nonpolypoid lesion, which corresponds to the endoscopic classification.[110] However, in this thesis both terms are used since articles were already published using the term flat adenoma.

Nonpolypoid adenomas can be classified endoscopically as well as histologically. As histological criterion is used that the height of nonpolypoid adenomas does not exceed twice the height of the surrounding normal mucosa; in practice this means less than 3 mm in height.[111] This definition, however, cannot be used during an endoscopy. Moreover it is also difficult to be applied by a pathologist due to fixation artifacts and, in slightly depressed lesions, the adjacent mucosa may be thinner than the normal epithelium.[110] Therefore it is recommended to classify nonpolypoid lesions endoscopically. Endoscopically, the height of the lesions can be estimated by using the forceps or the mini-snare. Nonpolypoid lesions are defined as lesions with a height less than half of the diameter.[112] This definition is simple and useful in routine practice. In practice, this often means that all lesions with a height smaller than 2-3 mm (measured using the forceps)[105] are classified as nonpolypoid as well as very broad lesions that are 5 mm in height.[104]

All superficial lesions (i.e. lesions that are limited to the superficial layers, mucosa and submucosa of the colorectal wall) can be further classified according to the Paris endoscopic classification of superficial neoplastic lesions.[113] This classification is based on the Japanese classification for gastrointestinal lesions[114] but has been simplified for practical reasons. The Paris classification makes a major division between polypoid (0-I) and nonpolypoid (0-II) morphology (Figure 1). The polypoid lesions can be subdivided in pedunculated or semipendunculated (0-Ip) and sessile (0-Is) morphology. Nonpolypoid lesions comprise slightly elevated (0-IIa), completely flat (0-IIb) and slightly depressed (0-IIc) lesions. Excavated or ulcerated superficial lesions (type 0-III) are practically nonexistent, and this type of lesion is described primarily in gastric cancer. Lesions that have penetrated into the muscularis propria or serosa are not classified as polypoid or nonpolypoid but separately as advanced[112,113] (Figure 1). In addition to the Paris classification, a distinction between small and large lesions can be made with all lesions larger than 10 mm classified as lateral spreading tumor (LST).[115] These LST can further be subdivided as lateral spreading tumors of the granular type (LST-G) and lateral spreading tumors of the non-granular type (LST-NG).[115]

Clinical relevance

Colonoscopy has a central role in every CRC prevention program, as it allows detection and removal of precursor lesions thereby successfully preventing CRC.[115,116] However, more recent studies showed that colonoscopy has its limitations in the prevention of CRC incidence and mortality, in particular for CRC of the proximal colon.[117-120] These limitations and the occurrence of post-colonoscopy CRCs (interval CRC) can be explained in two ways; 1) failure to detect or completely remove colorectal

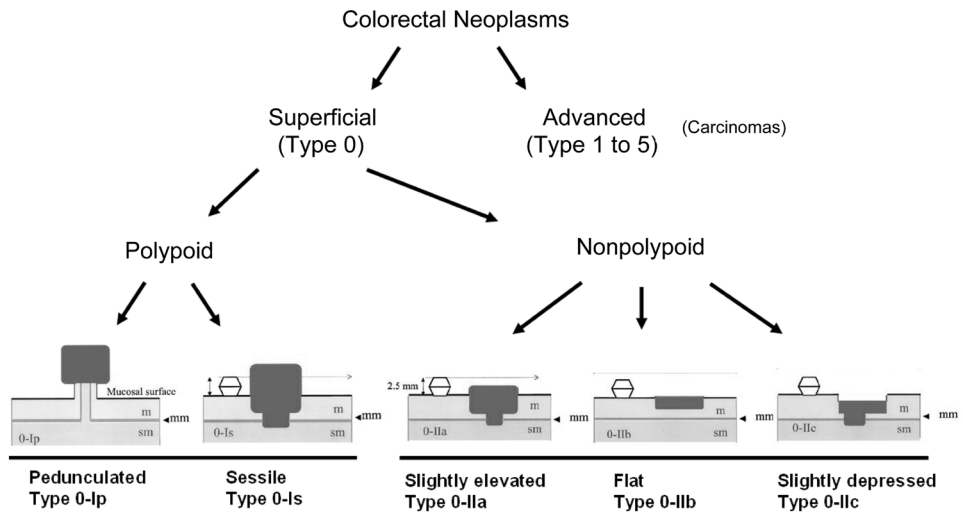

Figure 1: Schematic representation of the Paris classification of the major variants of type 0 neoplastic lesions. A lesion is considered nonpolypoid when lower than the height of the closed cups of a biopsy forceps (2.5 mm) m: mucosa, mm: muscularis mucosae; sm: submucosa. Figure adapted from[113,114]

neoplasms during a colonoscopy or 2) a more aggressive behavior (different tumor biology) of these interval cancers.[121,122] Nonpolypoid lesions have been suspected to be an important cause of these interval cancers[100,121] for two main reasons. First, as discussed above, nonpolypoid lesions may easily go undetected during colonoscopy (in particular by an inexperienced endoscopist and suboptimal bowel preparation). The second issue is that this different phenotype may be associated with a different underlying biology. At least a sub-group of nonpolypoid lesions have been associated with a more aggressive behavior and are considered to more likely contain advanced histology.[100,111,123] Especially subtype 0-IIc has an increased risk to contain high grade dyspasia (HGD) or invasive cancer at the time of diagnosis. In line with several other studies,[101,108,114,124] a recent study by Moss *et al*.[125] found that 31.8% of the depressed lesions and 15.3% of the non-granular lateral spreading type (LST-NG) contain submucosal invasion, indicating a potentially more aggressive biology of these lesions. For these reasons, it has been suggested that nonpolypoid lesions would have an accelerated progression from adenoma to carcinoma (e.g. they would complete the adenoma to carcinoma sequence at higher speed than polypoid adenomas).[100,111,114,123] If this proves to be true, apart from improving the GI endoscopy educational programs, more frequent surveillance may be needed in patients with nonpolypoid lesions to prevent interval cancers.

Molecular characteristics

Adenomas are the precursor lesions of carcinomas, however it has been estimated that only about five percent of all adenomas eventually evolve into CRC.[11] Classical characteristics (e.g. size, villous histology and grade of dysplasia) fail to accurately distinguish between adenomas that do progress to a carcinoma and those that will not.[12-14] While formal estimations of progression risk would require longitudinal studies leaving the adenomas *in situ*, which therefore are unethical and will never be conducted to the full extent necessary to answer the question, determining biological features that underlie this progression is feasible. Understanding the biology of colorectal cancer development will help to better recognize adenomas that will become malignant.[126] More extensive characterization on the molecular traits of nonpolypoid lesions compared to polypoid ones could further help to understand whether nonpolypoid lesions truly represent a different biological entity.

In the past, molecular studies have been initiated to investigate the tumor biology of colorectal nonpolypoid lesions. Interestingly, initial molecular studies have indicated a lower incidence of *KRAS* mutations in nonpolypoid adenomas,[127,128] while more recent studies contradict these findings.[129,130] Further contradicting results have been reported for other events, like *BRAF* mutation.[130,131] This controversy may be due to methodological issues including small sample sizes, heterogeneous definition of nonpolypoid adenomas and selection bias. Regarding other molecular events, such as the involvement of MSI, CIN or CIMP, no or only little research has been performed, mostly on datasets of limited size. As a consequence, at the start of this project still little was known about the molecular events in nonpolypoid colorectal lesions.

AIM AND OUTLINE OF THIS THESIS

Aim

As tumor phenotypes, for a substantial part, are driven by their genotypes, we aimed to investigate the molecular characteristics of nonpolypoid adenomas. As described above, little is known about the molecular characteristics of nonpolypoid adenomas, therefore the aim of this thesis was to investigate the molecular characteristics of nonpolypoid adenomas, thereby providing knowledge whether and how these lesions differ from regular polypoid lesions.

Outline

After identifying the molecular events that are of interest to investigate, we collected a large series of well-characterized nonpolypoid adenomas and polypoid adenomas. These were used to answer the following questions.

What is currently known about molecular changes in nonpolypoid adenomas?

In **Chapter 2**, we conducted a systematic search on studies that investigated molecular characteristics of nonpolypoid lesions. We conducted a systematic search on all studies that investigated the *KRAS*,

BRAF or *APC* mutation status, MSI or CIMP status or methylation of single genes in NP-CRNs. This resulted in a comprehensive overview of what currently is known about the molecular changes in nonpolypoid lesions. Furthermore, meta-analyses were performed for all studies investigating *KRAS, APC* or *BRAF* mutation status and MSI status, suggesting that nonpolypoid lesion might have a distinct molecular pathway. However, these meta-analyses also showed a large heterogeneity amount the different studies. The exact cause of this large heterogeneity could not be established but many studies pooled different nonpolypoid subtypes and/or histological types (i.e. adenomas and carcinomas). Furthermore many different definitions of nonpolypoid lesions where used. This study identified the gaps in our knowledge on molecular changes in nonpolypoid lesions and helped us to identify new interesting areas on molecular research in this field. The most important one was the need for studies that contain a large amount of well-characterized nonpolypoid lesions.

Is the mutation frequency for known CRC genes different between polypoid and nonpolypoid adenomas?

Our sample series was used to analyze the mutation status of both already investigated genes, such as *KRAS*, *BRAF* and *APC*, and a number of other genes known to be mutated in CRC, but never investigated before in nonpolypoid adenomas such as *NRAS*, *FBXW7*, *PIK3CA*, *PTEN* and *CTNNB1*. In total 14 genes were comprehensively investigated as is described in **Chapter 3**. Contradicting previous studies we did not observe a difference in *KRAS* mutation frequency. However, we did observe significantly less *APC* mutations in nonpolypoid adenomas compared to polypoid adenomas.

What is the occurrence of CIN and MSI in nonpolypoid adenomas?

Chromosomal alterations are known to play a role in CRC carcinogenesis, however, the number of studies investigating this in nonpolypoid lesions is lagging behind. We used a large number of nonpolypoid and polypoid adenomas and investigated DNA copy number alterations using a high resolution arrayCGH platform. In parallel the MSI status of both groups was investigated. The results of this study are described in **Chapter 4**. We found significantly more chromosome 5q (locus of *APC*) loss and simultaneously less APC mutations in nonpolypoid adenomas compared to polypoid adenomas. This could suggest a different mechanism to disrupt the Wnt-signaling pathway. The occurrence of MSI in nonpolypoid adenomas was very low, similar to that of polypoid adenomas.

Are there differently methylated genes in nonpolypoid adenomas compared to polypoid ones and what is the occurrence of CIMP?

Studies that investigate epigenetic events in nonpolypoid adenomas are scarce. Only a few studies investigated the DNA promoter hypermethylation of a few genes. Consequently, the role of methylation in nonpolypoid colorectal adenomas is still largely unexplored. In **Chapter 5** we investigated the methylation status of 11 genes known to be methylated in CRC. In addition we also investigated the CIMP-status in nonpolypoid and polypoid adenomas. To validate the results,

two independent cohorts were used (each containing around 200 adenomas, ~100 nonpolypoid adenomas and ~100 polypoid adenomas). We found no differences for the investigated genes between nonpolypoid and polypoid adenomas that could be observed in both cohorts. Also the CIMP status was not different between both phenotypes.

Are there difference between nonpolypoid and polypoid adenomas concerning DNA promoter hypermethylation of genes involved in the Wnt-signalling pathway?

In **Chapter 6** we specifically investigated promoter hypermethylation of genes which are involved in the Wnt-signaling pathway in nonpolypoid adenomas. This pathway is an important pathway in CRC and can be affected in many different ways. In this study we combined our results with previously obtained results on *APC* mutation and chromosome 5q (locus of *APC*). We observed less methylation of *WIF1* and *DKK3* in nonpolypoid compared to polypoid adenomas.

Chapter 7 addresses the influence of MSI on the survival of CRC patients that were included in the Active specific immunotherapy (ASI) trial.[132] This trial consistent of an autologous tumor cell vaccine given as adjuvant treatment and has been shown to improve recurrence-free survival of patients with colon cancer. The aim of this study was to investigate if the beneficial effect of the ASI given as adjuvant treatment was related to MSI. We observed that patients with MSS Dukes B tumors who received ASI treatment showed a significantly improved recurrence-free survival compared with controls.

Finally, **Chapter 8** provides a summary of all the studies included in this thesis. General conclusions and a discussion on further research involving nonpolypoid adenomas are presented.

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